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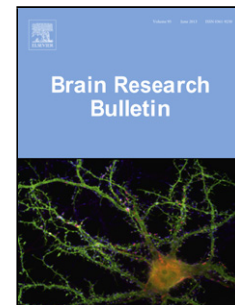
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**a Single Low Dose of Valproic Acid in Late Prenatal Life Alters Postnatal
Behavior and Glutamic Acid Decarboxylase Levels in the Mouse.**

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Abstract

Rationale Rodents exposed to valproic acid (VPA) in prenatal life exhibit post-natal characteristics analogous to autism spectrum disorder (ASD). Many previous studies used relatively high doses of VPA during early pregnancy, potentially confounding interpretation because the offspring are the ‘survivors’ of a toxic insult. Low dose or late gestation exposure has not been widely studied. *Objectives* We examined the behavioral sequelae of late gestation exposure to low dose VPA in the mouse. We also examined postnatal levels of glutamic acid decarboxylase (GAD65 and GAD67) as markers for GABA neurons, because GABA pathology and subsequent excitatory/inhibitory imbalance is strongly implicated in ASD. *Methods* Pregnant C57BL/6N mice received a single subcutaneous injection of 100 or 200 mg/kg on gestation day 17. The control group received a saline injection on the same day. The offspring were tested in a battery of behavioral tests in adolescence and adulthood. Six brain regions were harvested and GAD65 and GAD67 were measured by western blotting. *Results* Compared to saline-exposed controls, adult mice exposed to prenatal VPA had impaired novel object exploration and fear conditioning anomalies. GAD67 was decreased in midbrain, olfactory bulb, prefrontal cortex and increased in cerebellum, hippocampus and striatum; GAD65 was decreased in all 6 regions. *Conclusions* Our results suggest that a low dose of VPA in late pregnancy has persistent effects on brain development, and in particular the GABA system, which

may be relevant to ASD. Further attention to the impact of gestation time and dose of exposure in VPA-induced ASD models is encouraged.

Keywords

valproic acid; valproate; autism spectrum disorder; ASD; mouse; glutamic acid
decarboxylase

1. Introduction

Exposure to valproic acid (VPA) in pregnancy is associated with a higher incidence of autism spectrum disorder (ASD) by case reports and population studies [1-9]. Rodents exposed to VPA prenatally simulate a number of behavioural traits found in ASD [10, 11]; eg. lower sensitivity to pain, increased repetitive/stereotypic-like activity, higher anxiety, decreased level of social interaction [12]. They have also been reported to recapitulate neurochemical and neuroanatomical features of ASD such as impaired inhibitory gamma amino butyric acid (GABA)-ergic synaptic transmission [13], increased frontal cortical serotonin [14], altered excitatory post-synaptic development [15]; and differences in brain structure and gene expression [16-19].

One of the challenges with this model is that it is difficult to translate the doses of VPA used in humans to the equivalent in rodent. In humans, VPA is used within its relatively narrow therapeutic range, for example 20-30 mg/kg in epilepsy[20]. In contrast, rodents have been reported to be relatively resistant to VPA toxicity [21] and the majority of the reports that studies that link VPA exposure in pregnancy to ASD-like outcomes in rodents, have used either a moderate dose of VPA (200 mg/kg) over consecutive days, or a single high dose of VPA (500-800 mg/kg). However, these regimes can induce physical malformations and even death of offspring [11, 22, 23]. Specifically, the usual rate of perinatal loss before the postnatal day 6 in rats is 8.9%; but this increases to 15.6% following administration of 300 mg/kg VPA, and to

48.7% following administration of 500 mg/kg VPA[24]. This raises a question about how best to interpret results from survivors of such significant insults. Despite, this potential confound, few studies have examined low dose exposure to VPA in pregnancy in experimental settings [25-27].

The gestational timepoint of exposure may also influence outcome following prenatal environmental exposures. For example, we and others have reported that exposure to maternal immune activation (MIA) in early or late gestation precipitates a distinct phenotype in terms of both nature and severity [28-30]. In the VPA model, the embryotoxicity of VPA initially came to light because it induced neural tube defects. As a result, many animal studies focused on the time of neural tube closure in early pregnancy [3, 31]. However, despite initial evidence that embryotoxicity following VPA exposure may be even higher in late gestation [32], the effects of late exposure VPA have received scant attention since.

Finally, another over-looked factor which may influence the outcome of exposure to VPA in prenatal life, is the sex of the offspring. The majority of VPA studies have focused on male off-spring only, by convention and/or the reported higher incidence of males with ASD [33].

Therefore, in this series of experiments we aimed to investigate the effects of low dose VPA (100 mg/kg or 200 mg/kg) administered to pregnant mice in the late

gestation. We examined juvenile and adult mice of both sexes in paradigms which measure behaviours relevant to ASD, namely open field activity, prepulse inhibition of startle (PPI) measure of sensorimotor gating, olfactory discrimination (adults only) and fear conditioning (adults only) paradigms. In addition, using western blot, we measured regional brain levels of Glutamic acid decarboxylase (GAD), the rate-limiting enzyme in the conversion of excitatory neurotransmitter glutamate to inhibitory GABA in the brain [34]. This is because brain excitatory/inhibitory imbalance is increasingly linked to ASD [35-38], and both GAD65 and GAD67 isoforms, have been reported to be altered in ASD [39], and also in preclinical models of ASD such as the BTBR mouse [40] and the MIA model [41]. However, it is unknown whether prenatal VPA exposure alters GAD level postnatally.

2. Materials and methods

2.1 Animals and sample collections

Twelve pregnant female C57BL/6N mice were supplied by HKU Laboratory Animal Unit (LAU) breeding colony - the day that a vaginal plug was identified was taken as embryonic day 0 (GD0). They were provided to the behavior unit within the LAU on GD15. The mice were maintained in a normal light cycle (lights on at 7:00; off at 19:00) through-out the entire experiment. The temperature and humidity in the holding room was controlled at $21\pm1^{\circ}\text{C}$, and $55\pm5\%$ respectively, and a conventional *ad libitum* food and water diet was supplied by the LAU. All experiments were

approved by the Committee on the Use of Live Animals in Teaching and Research at The University of Hong Kong, and every effort was made to minimize the number of animals used and their suffering.

Valproic acid was purchased as sodium valproate (Sigma, St Louis, USA) and was dissolved in 0.9% saline to achieve a concentration of 20 mg/ml. Twelve pregnant mice were divided equally into 3 weight-matched groups and injected with 0.9% saline control (CON-0), 100 mg/kg VPA (VPA-100), or 200 mg/kg VPA (VPA-200) on GD17. Pregnant mice were housed individually and allowed to raise their own litters. Eighty mice were born in total. There were 13 female and 17 male in CON-0 group, 14 female and 13 male in VPA-100 group, and 8 female and 15 male in VPA-200 group. Offspring were weaned on postnatal day 21 and mice of the same sex from the same litter were group housed in standard cages. No more than four animals were kept to a cage. All the mice were generally healthy and careful visual inspection revealed no abnormal physical features.

The mice were weighed twice, once as juveniles in week 4 right after weaning and once in week 12 as adults. The open field test, the novel object recognition test and the prepulse inhibition (PPI) test were conducted at ages 5-6 weeks and 12-15 weeks. Olfactory testing was conducted only in adulthood (week 16) because juvenile mice are known to respond unpredictably to odors [42]. The fear conditioning test was also only conducted once at the end of behavior tests (week 17), to avoid any residual

influence of the aversive nature of that test. MRI scan of mouse brain was conducted in 7 female and 7 male from each treatment group in week 19 (data under analysis and not reported here). After an undisturbed period all mice were sacrificed in week 22. Their brains were harvested and rapidly dissected to collect tissue samples from cerebellum (CB), hippocampus (HP), midbrain (MB), olfactory bulb (OB), prefrontal cortex (PFC) and striatum (STR).

2.2 Behavioral tests

2.2.1 Open field test

The open field test was used to measure locomotor activity in the dim light and general anxiety levels in rodents in the bright light [43, 44]. The experimental setting comprised four identical square arenas (40×40 cm) surrounded on all sides by a 40-cm white plastic wall. A central area (15×15 cm) was drawn in the center of each arena. A digital camera was mounted above, capturing images from all four arenas at the same time and the videos were analyzed by the EthoVision tracking system (Version 3.1, Noldus Information Technology). The mice were tested in squads of four. They were gently placed in the center of the appropriate arena in the dim light and allowed to explore undisturbed for 30-min. Locomotor activity was indexed by distance travelled recorded in successive 5-min bins and the anxiety-related behaviors were measured by distance travelled in the central area.

2.2.2 Novel object Recognition test

The procedure was adapted from previous protocols [45, 46]. A plastic cage (30 × 30 × 30 cm) with white walls was used in 3 sessions. In the habituation session on the first day, the mouse was placed into the empty cage to habituate for 10-min. On the second day, a 10-min training session preceded the 5-min test session by 1 hour. During the training session, two identical objects A (blue cylinder, 3 cm diameter × 1 cm height,) were placed near the corners of one wall of the cage (5cm from each adjacent wall). The mouse was placed near the opposite wall, facing it, then allowed to explore both objects. During the test session, a fresh “familiar” object A’ and a novel object B (pink cuboid, 3 × 1 × 1 cm,) were placed in the same place as objects in the training session and the mouse was allowed to explore. To avoid odor clues, the cage and objects were thoroughly cleaned with 70% ethanol between mice. The videos of the test phase were analyzed using the EthoVision tracking system and ‘Exploration’ was defined as the nose of mouse placed within 1 cm of the object A (T_A) and the object B (T_B). A discrimination index (DI) was calculated as: $DI = (T_B - T_A) / (T_B + T_A) \times 100$

2.3.3 Prepulse inhibition (PPI) test

The procedures and testing parameters for the PPI test used here followed those described previously [29]. Two standard acoustic startle chambers for mice (SR-LAB, San Diego Instruments, San Diego, CA, USA) generated the continuous background

noise of 65 dB and white noise stimuli. The white noise stimuli was set to be 100 dB, 110 dB and 120 dB as three levels of pulse, and 71 dB, 77 dB and 83 dB as three levels of prepulse (+6 dB, +12 dB, +18 dB) over the background noise). The whole body response was transformed to an analogue signal by a piezoelectric unit then digitized and stored. PPI was calculated as the reduction of startle responses in the prepulse-plus-pulse trials relative to startle in pulse-alone trials. % PPI = (pulse-alone - prepulse-plus-pulse)/pulse-alone \times 100%.

2.2.4 Olfactory test

The protocol was adapted from that described previously [47]. The test apparatus was a rectangle box (30 \times 30 \times 30 cm) with a hanging holder in the center. The subject mouse was put into the box, with a dry cotton tip held in the holder. The animal was allowed to habituate to the setting for 2-min (Trial 0). The dry cotton tip was then exchanged for a series of tips soaked with different liquids. There were 15 continuous 2-min trials as shown in Table 2.2. In trial 1-3 water was used to establish baseline activity. Trials 4-6 and 7-9, saturated solution of commercial milk tea powder and Commercial strawberry jam were used to investigate the responses to 2 non-social odors. In Trials 10-12 and 13-15, 2 mixed urine dilutions (1:100) were used as social odors. The urine mixes were collected each from 3 different mice held in a separate holding room. Urine from mice of the same sex as the subject mouse was always used.

The time and frequency that each mouse sniffed each cotton tip was recorded to index odor-attraction; the distance moved within each trial was also recorded.

2.2.5 Fear-conditioning test

The paradigm is based on the phenomenon that organisms can establish an association between a neutral environmental cue, the conditioned stimulus (CS), with an inescapable foot-shock, the unconditioned stimulus (US), when these two stimuli are closely paired. When conditioning is established, the CS alone can elicit fear [48].

The procedure was adapted from previous studies [49-51]. The apparatus comprised two identical conditioning chambers (25 × 25 × 25 cm each). The front side was a fold-down door and the other three sides of the chambers were made from removable panels that slid onto rails. The wall panels were white color on one side and had patterns on the other side. The floor was composed of 4-mm steel rods placed 8.9-mm apart that connected to a computer-controlled ‘foot-shock’ administrator. The chambers were entirely encased within a sound attenuating box with a door at the front. On the roof of the sound proofed box was a single white light bulb, an extractor fan, a black-and-white camera and a tone generator.

The fear conditioning test was conducted over 3 days. Day 1 was the training session of 13.5-min. A mouse was placed into a conditioning chamber with the white side of wall panels facing inside. It was allowed to explore the chamber freely for 6-min

before 3 paired presentations of a clicker as the CS (30s, 4 Hz, 80 dB) and footshock as the US (2s, 0.5 mA) were presented. The inter-pair interval was 2-min with 2-min rest after the final clicker/shock pairing in the chamber. The chambers were cleaned with 70% ethanol between each training session. Day 2 was a context extinction test of 8-min. Each mouse was placed the same chamber used for training on Day 1 with no clicker and no foot shock. Their activity was measured. The floor was cleaned with 70% alcohol between each subject. Day 3 was a cue test of 13.5-min. Each mouse was placed in a 'novel' environment (the side wall panels were reversed so that the patterned surface faced inside and the grid floor was changed to a white flat panel). The first 6-min measured exploratory activity in novel context. And during the second 6-min of the session, the clicker was played as in the training session, but without the foot shock. Between each session the floor was cleaned with Hibiscrub to remove olfactory cues from the alcohol used during training period.

Freezing response (absence of movement except respiratory movement) was measured using the EthoVision tracking system and compared across groups in 5 categories: pre-shock (the first 6-min of Day 1), associative learning (the last 7.5-min of Day 1 test), context conditioning (8-min of Day 2 test), response to novel environment (the first 6-min of Day 3 test, to ensure there were no residual context cues available) and cue conditioning (the last 7.5-min of Day 3 test, response to CS alone).

2.3 Western blot for GAD65 and GAD67

The brain tissues were homogenized at 4°C in lysis buffer (1:5, wt/vol) containing 1 mM EDTA and 20 mM phenylmethanesulphonyl fluoride. The proteins from resultant supernatant were determined (Bio-Rad Protein Assay) for Sodium Dodecyl-sulphate Polyacrylamide gel electrophoresis (SDS-PAGE). Proteins (20 µg / lane) were subjected to electrophoresis on a 12% (wt/vol) polyacrylamide gel in SDS, and gels subsequently processed for electroblotting to polyvinylidene difluoride (PVDF) membranes. The blotted PVDF membranes were saturated with 5% (wt/vol) of skimmed milk in Tris Buffer Saline, pH 7.4 and 0.1% (vol/vol) of Tween 20 for 1 h at room temperature. The membranes were sequentially incubated with primary antibodies to the following proteins: GAD65 (rabbit polyclonal IgG antibody, 1:2000 dilution, Abcam) or GAD67 (rabbit polyclonal IgG antibody, 1:2000 dilution, Abcam) for 1 h at room temperature. After thorough washing, the positive bands were revealed using ECL western blotting detection reagents and autoradiography film (Amersham, Biosciences, UK). The intensities of the bands were quantified using ImageJ software [52].

2.4 Statistics

Data from the behavioral tests and western blot test were analyzed using a General Linear Model (GLM) in SPSS software with fixed factors Drug (saline, 100 mg/kg VPA, 200 mg/kg VPA) and Sex (female vs. male). For the tests that carried out twice

(weights, open field, novel object exploration and PPI), a repeated measures GLM included Age (juvenile vs. adulthood). Significant main effects of Drug were further explored using post-hoc Dunnett's test for the 3 levels of Drug. Significant interactions of Sex \times Drug and/or Age \times Drug were further explored by studying Drug effects in each sex and age subgroup. Chi-square test was used to compare the numbers of mice excluded from each group in the novel object recognition task. Pearson bivariate correlate analyses were used to investigate the relationship between cue and contextual conditioning in each group. Any difference in correlation coefficients was tested by conversion to z-scores (<http://www.vassarstats.net/rdiff.html>) [53]. To screen for potential outliers using a box plot approach, the relationship between cue and context conditioning was indexed as [%freezing cue conditioning - %freezing context]. A threshold of $p < 0.05$ (2-sided) was considered to be statistically significant. All data are presented as mean \pm S.E.M.

3. Results

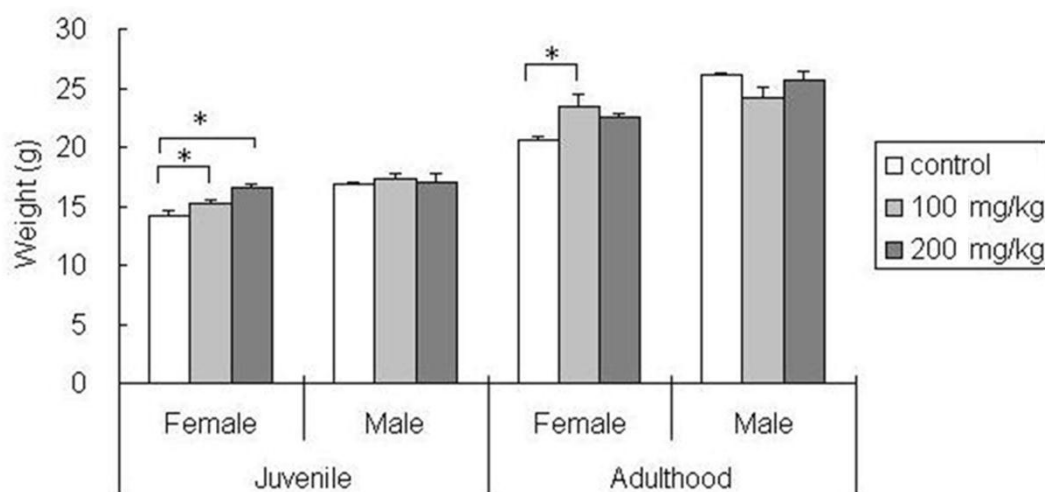
3.1 Physical features

The number of offspring per litter and ratio of female to male was not different between the 3 groups (data not shown). VPA-100 mice and VPA-200 mice had no visible physical abnormalities. There was no significant main effect of Drug on the body weight. As expected males were heavier than females (Sex, $F_{(1, 32)} = 35.511$, $p < 0.05$); adults were heavier than juveniles, (Age, $F_{(1, 74)} = 867.251$, $p < 0.05$). A

significant Sex×Drug interaction ($F_{(2, 74)} = 4.266, p < 0.05$) was explained because female VPA-treated mice, not males, were heavier than the corresponding controls ($F_{(2, 32)} = 6.232, p < 0.05$) in both the VPA-100 and VPA-200 dose conditions (Figure 1).

Figure 1. The body weight of the mice.

The data are presented as means \pm S.E.M. *: Statistical significance ($p < 0.05$)



3.2 Open field test

There was no significant main effect of Drug or Sex. However, the effect of Age was significant - adult mice were more active than juveniles in all groups (Age, $F_{(1, 74)} = 8.583$, $p < 0.05$). There were no significant interactions with Drug on these measures.

3.3 Novel object recognition test

The mice that did not explore both objects ($T_B + T_A = 0$) either as juvenile or as adult were excluded from the repeated measures analysis of DI. Although a greater proportion of VPA mice were excluded than controls, this was not statistically significant. There was no significant difference between the numbers excluded from each group ($p > 0.05$) (Table 1). VPA treatment lowered DI as shown by a significant

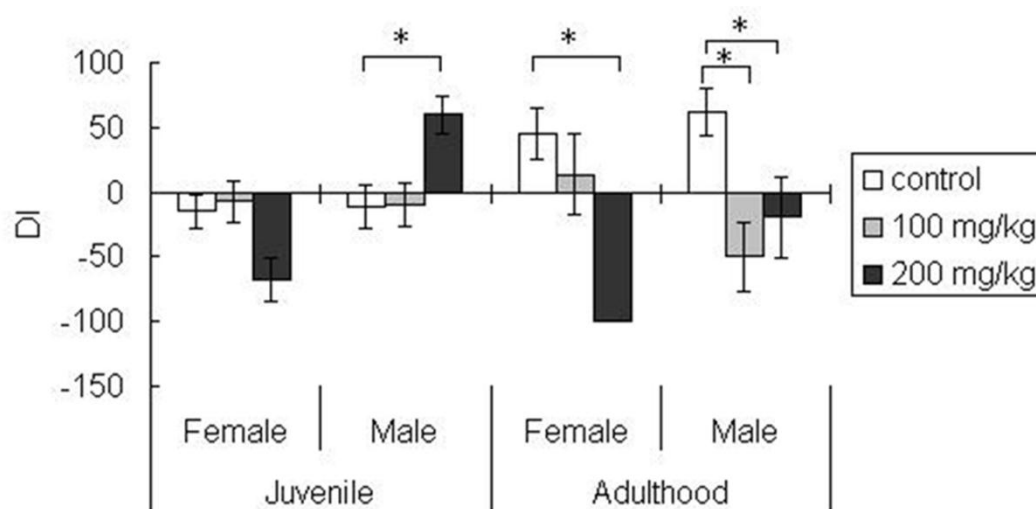
main effect of Drug on DI ($F_{(2, 45)} = 4.344, p < 0.05$). There were no main effects of Sex or Age, but significant Sex \times Drug ($F_{(2, 45)} = 6.109, p < 0.05$) and Age \times Drug ($F_{(2, 45)} = 7.670, p < 0.05$) interactions. Post-hoc testing confirmed that VPA lowered DI in both sexes in adulthood but not in juvenile males exposed to VPA-200 (Figure 2).

Table 1. The numbers of mice excluded from the DI calculation.

		Tested	Excluded
CON-0	Female	13	4(30.8%)
	Male	17	5(29.4%)
VPA-100	Female	14	5(35.7%)
	Male	13	3(23.1%)
VPA-200	Female	8	5(62.5%)
	Male	15	7(26.7%)

Figure 2. The discrimination index (DI) of the mice in the novel object recognition.

The data are presented as means±S.E.M. *: Statistical significance ($p < 0.05$)



3.4 Prepulse inhibition test

Startle reactivity

There was no significant main effect of Drug or Age on startle reactivity. Male mice had higher startle responses than females ($F_{(1, 74)} = 13.979, p < 0.05$) and louder Pulse caused greater startle ($F_{(2, 148)} = 607.059, p < 0.05$). There were no significant interactions with Drug.

PPI

There was no significant effect of Drug, Age or Prepulse on PPI. However, female mice had higher PPI than males ($F_{(1, 74)} = 7.241, p < 0.05$) and PPI was greater in louder Pulse conditions ($F_{(1, 74)} = 7.241, p < 0.05$). There were no statistically significant interactions with Drug.

3.5 Olfactory test

Sex dimorphism in the detection and/or processing of volatile urinary odors in mouse has been reported previously and, in particular, females are more sensitive to dilute urine samples [54]. Therefore, data from female and male mice in the olfactory test were analyzed separately. There was no significant difference between VPA-treated mice and the control mice of the same sex in total distance moved in this test, baseline activity (trial 1-3), response to non-social odors (trial 4-9) and response to social

odors (trial 10-15). The response to odors depended on the sequence that an odor was used; all three groups explored the cotton tips more when a new odor was presented, showing that they could distinguish the new odor from the former one ($F_{(2, 1152)} = 46.681, p < 0.05$).

3.6 Fear conditioning test

There was no significant main effect of Drug or Sex, and no significant interaction in measures of freezing during pre-shock, associative learning, context conditioning test, response to novel environment and cue conditioning test. All the 3 groups showed a higher level of freezing in the context test ($F_{(1, 122)} = 447.511, p < 0.05$) and the cue test ($F_{(1, 145)} = 61.793, p < 0.05$) relative to the pre-shock/pre-conditioning period, indicating that fear conditioning had taken place (Table 2).

The relationship between context and cue conditioning was explored using Pearson correlations. In CON-0 mice, as expected, there was a significant negative relationship ($r = -0.515, p = 0.020$); that is, stronger cue conditioning was associated with weaker context conditioning and vice versa. But this did not exist in VPA-100 mice ($r = -0.027, p = 0.906$), and was nearly (not reach the significance level) reversed in VPA-200 mice ($r = 0.506, p = 0.093$); that is, stronger cue conditioning was associated with stronger context conditioning (Figure 3A). One outlier in the VPA-100 group was identified. However excluding data from this animal did not materially alter the results (Figure 3B). Moreover, there was a significant difference

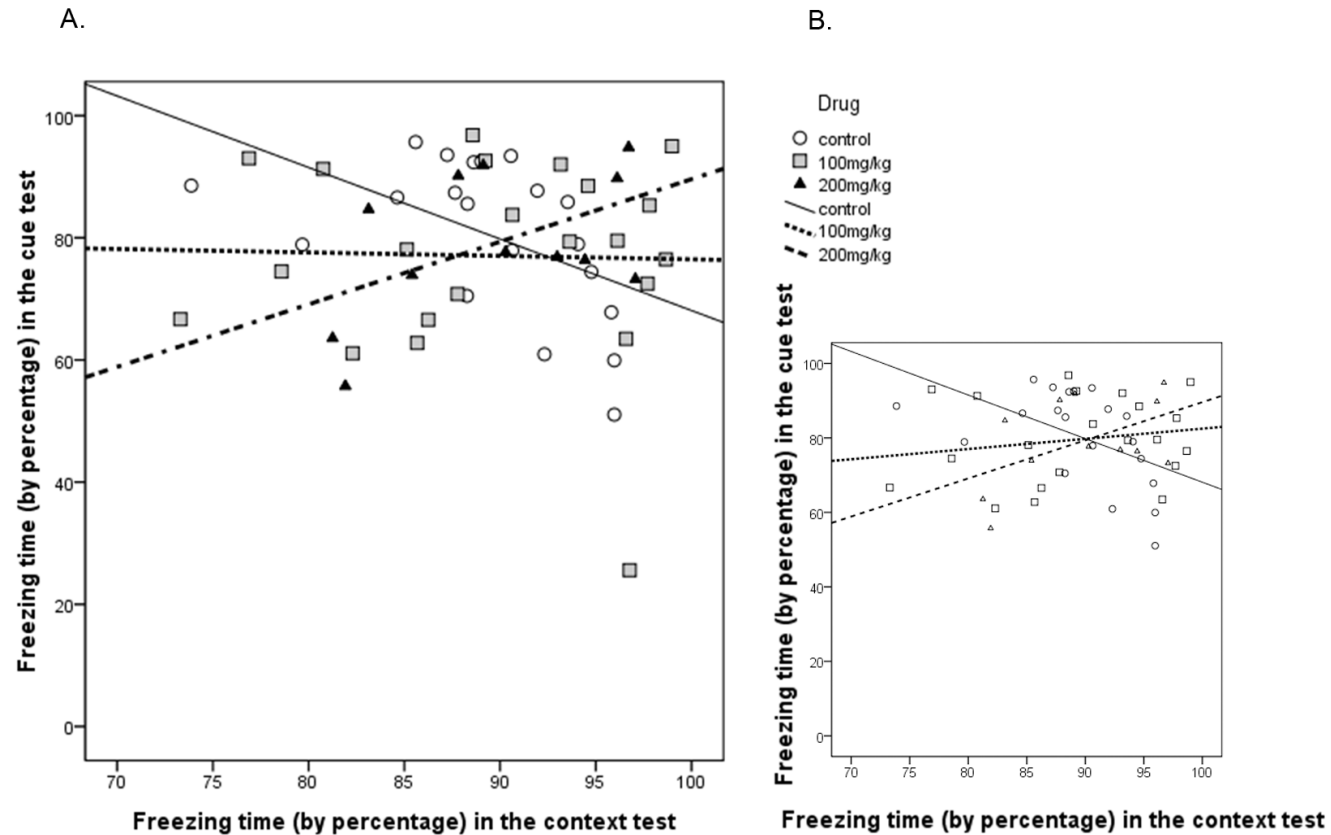
between correlation co-efficients in CON-0 mice and VPA-200 exposed groups ($Z = -2.76, p < 0.05$).

Table 2. The performance of mice in the fear conditioning test.

The data are presented as mean \pm S.E.M.

	Control			100 mg/kg			200 mg/kg		
	Total	Female	Male	Total	Female	Male	Total	Female	Male
pre-shock	57.57 \pm 1.724	58.80 \pm 1.883	56.91 \pm 2.486	58.65 \pm 1.543	57.72 \pm 2.150	60.27 \pm 2.005	61.84 \pm 1.659	63.49 \pm 1.340	58.54 \pm 4.053
context	89.42 \pm 1.254	89.06 \pm 2.927	89.61 \pm 1.230	89.51 \pm 1.633	87.45 \pm 2.317	93.11 \pm 1.282	89.69 \pm 1.687	89.86 \pm 2.130	89.34 \pm 3.173
Cue	80.48 \pm 2.852	81.42 \pm 5.811	79.98 \pm 3.266	77.07 \pm 3.441	75.83 \pm 5.072	79.24 \pm 3.606	79.04 \pm 3.415	77.57 \pm 3.937	81.97 \pm 7.187

Figure 3. The relationship between context and cue conditioning.



A. The relationship between context and cue conditioning of the three groups. In CON-0 mice, as expected, there was a significant negative relationship; That is, the more the animals learned that the explicit cue (the clicker) predicted a foot shock, the less they learned about the contextual stimuli (the panel setting of the box) to the foot shock. (shown by the solid line). But this did not exist in VPA-100 mice (shown by

the dotted line), and was nearly reversed in VPA-200 mice (shown by the dash line); that is, VPA-200 mice learn ‘indiscriminately’ and form a relationship between the explicit or contextual cues to the foot shock equally.

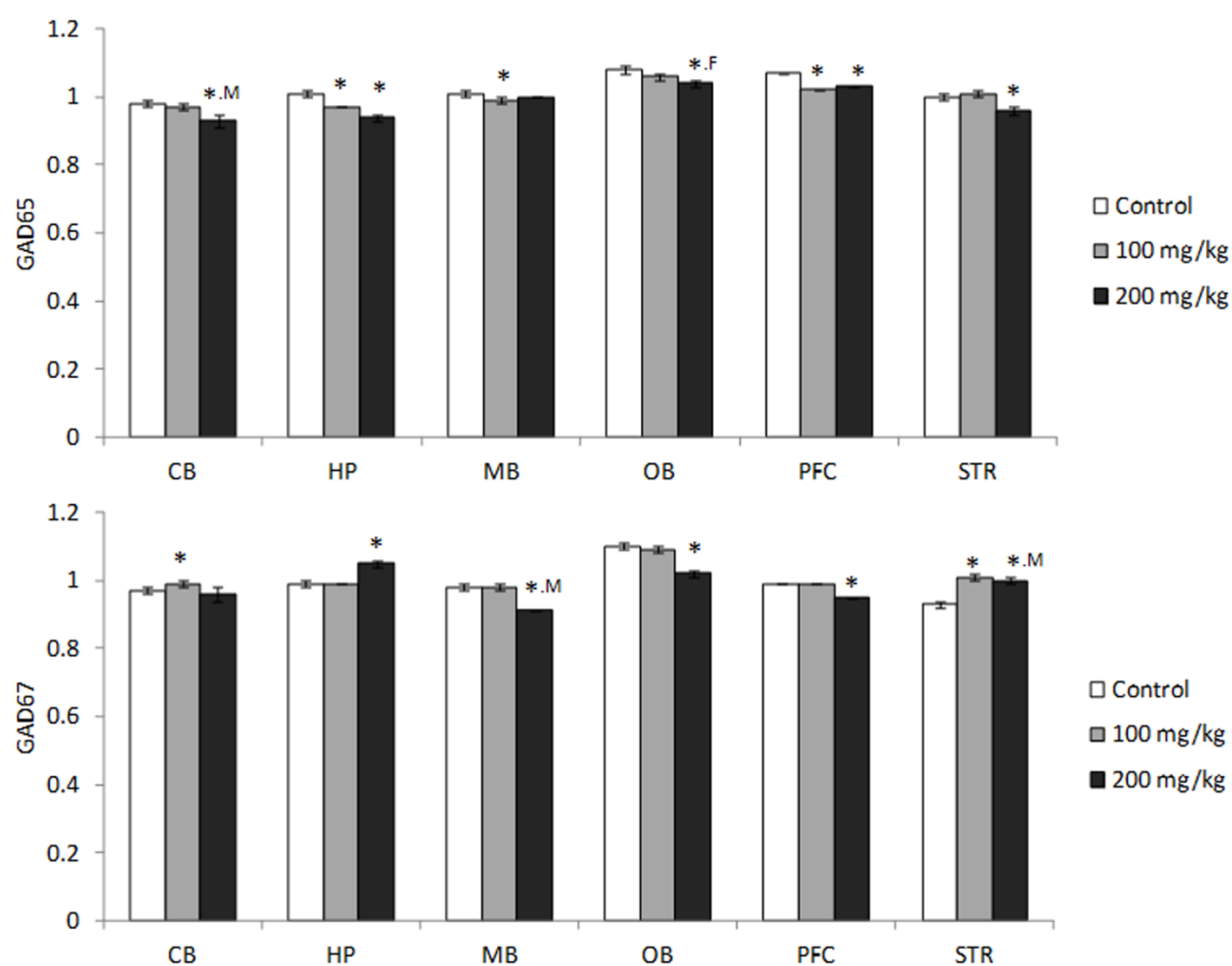
B. After deleting one outlier in the VPA-100 group that was identified in A, the results did not alter materially.

3.7 GAD65 and GAD67 Western Blot analysis

GAD65 in VPA-treated groups was significantly lower than the control group in all brain areas sampled: CB ($F_{(2, 36)}=9.046, p < 0.05$), HP ($F_{(2, 36)}=26.873, p < 0.05$), MB ($F_{(2, 36)}=4.700, p < 0.05$), OB ($F_{(2, 36)}=4.186, p < 0.05$), PFC ($F_{(2, 36)}=31.179, p < 0.05$), STR ($F_{(2, 36)}=7.210, p < 0.05$). The effect in the majority of regions was independent of Sex, with the exception of a significant Drug \times Sex interaction in CB ($F_{(2, 36)}=8.816, p < 0.05$) - VPA exposed males had lowest levels of GAD65; and in OB ($F_{(2, 36)}=3.613, p < 0.05$) - VPA-exposed females had lowest levels of GAD65 (Figure 4).

GAD67 in VPA-treated groups was significantly lower than the control group in MB ($F_{(2, 36)}=41.445, p < 0.05$), OB ($F_{(2, 36)}=13.492, p < 0.05$) and PFC ($F_{(2, 36)}=13.267, p < 0.05$), and was higher in CB ($F_{(2, 36)}=5.615, p < 0.05$), HP ($F_{(2, 36)}=34.435, p < 0.05$) and STR ($F_{(2, 36)}=7.210, p < 0.05$). Again in most regions this was independent of Sex; exceptions were a significant Drug \times Sex interaction in MB and STR, where GAD67 differences were most prominent in male VPA-treated mice (Figure 4).

Figure 4. GAD65 and GAD67 in different brain areas



*Statistically significance difference compared to control group post-hoc ($p < 0.05$).

^M Sex*Drug interaction - significant difference driven by male VPA mice.

^F Sex*Drug interaction - significant difference driven by female VPA mice.

4. Discussion

The present study demonstrated that a single low dose of VPA in late gestation disrupted postnatal behavior and altered the levels of GAD65 and GAD67 in multiple brain regions of the offspring exposed. Mice exposed to low dose VPA in late embryonic life showed subtle but significant changes in stimulus processing underlying response to novelty or associative learning. The behavioral phenotype was partly influenced by sex and postnatal age of testing. However, compared to the saline control group, exposure to 200 mg/kg VPA did not invariably result in greater differences than 100 mg/kg VPA. The possibility that the VPA behavioral phenotype may be at least partly explained by a GABA system abnormality was signaled by abnormalities in regional GAD levels in VPA exposed groups.

Behaviors altered by a low dose of VPA in late gestation

Rodents exposed to VPA prenatally have been reported to simulate a number of traits found in ASD and this is consequently considered to be a promising animal model of aspects of the human condition [10, 11]. However, the dose regimes used (either a moderate dose over consecutive days, or a single high dose of VPA) may induce physical malformations and even death of offspring [11, 22, 23], and thus complicate the interpretation of the results generated from ‘survivors’ of such a toxic insult. In addition, despite evidence that the timing of a gestational exposure may influence the outcome of offspring [26], this has been largely overlooked. Here, we found that mice

exposed to a low dose of VPA in late gestation did not cause embryonic death, or obvious congenital abnormalities, but did alter stimulus processing in both the novel object test and the fear conditioning paradigm, indicating that prenatal exposure to VPA causes persistent changes in response to the external environment. This is broadly in line with studies of sensory processing in ASD which report impaired sensory discrimination [55], as well as dysmodulation of sensory input and motor output [56, 57].

Novel object recognition: The lower DI observed in VPA exposed animals could possibly be due to ‘memory’ problems, that is, the VPA-treated mice did not ‘remember’ the familiar objects and treated both objects in the test phase as novel. However, VPA exposed animals subsequently ‘remembered’ the cue and contextual stimuli paired with a foot shock in the fear conditioning test, arguing against a general memory difficulty. Alternatively, the result could be explained by a ‘dislike’ of novelty. The higher rate of exclusion of VPA animals due to non-exploration is consistent with this explanation, though this did not reach statistical significance. ‘Avoidance’ is a feature of ASD. For example, as early as 1982, Kootz et al. noted that “rather than responding to novelty with orientation, observation, and exploration, the autistic child often responds with avoidance” [58]. Dislike of novelty is also reflected in an excessive adherence to routines or excessive resistance to change, one of the core symptoms of ASD [59].

Fear conditioning: In the fear-conditioning test there is learning about a ‘simple’ or discrete cue (day 3), and learning about the contextual stimuli (day 2) [60]. This test revealed that VPA-exposed animals had clear differences from the control animals in the way they link explicit and contextual stimuli to a foot shock. In the control group, cue and contextual conditioning were negatively correlated. That is, the more the animals learn that the explicit cue (the clicker) predicts a foot shock, the less they will learn about the contextual stimuli (the panel setting of the box) to the foot shock. This pattern is predicted by animal learning theory, i.e. learning less about CS allows greater learning about context and vice versa [61]. This relationship did not exist in VPA-100 and was almost reversed in VPA-200 mice. Thus, VPA mice appeared to learn equally well about an explicit cue and contextual cues in the experimental set-up. Therefore, VPA animals appear to ‘indiscriminately’ form a relationship between explicit or contextual cues and the foot shock. This may parallel the sometimes strong or unusual attachments or aversions to stimuli developed by a person with ASD; attachments which would not usually be formed by a person without ASD.

Associative learning is governed by amygdala and hippocampal circuits [62]. In particular, the hippocampus is thought to regulate processing of contextual cues [63, 64]. Thus ‘non-selective’ associative learning by VPA exposed animals may be underpinned by some ‘disconnect’ between amygdala and hippocampus hubs involved in stimulus processing. This potentially fits with reports that ASD-related difficulties in discriminating between conditioned and unconditioned fear stimuli stem

from “poor connectivity” between the amygdala and functional related cortical regions [65]; which is potentially due to a disruption in GABAergic signaling [66]. For example, a GAD65 knock-out has been reported to cause a generalization of cued fear (similar to that reported here) and impaired extinction of cued fear [66]; and our VPA exposed animals had significant reduction in GAD65 levels throughout the brain, including the hippocampus. Our results are also broadly consistent with observations by Markram and colleagues, who found that VPA-treated rats exhibited abnormally high and longer lasting fear memories [67]. However, since that team did not explore the relationship between CS and context conditioning, and we did not examine fear memories over a longer period, it is difficult to directly compare our 2 studies [67].

Behaviors unchanged by a low dose of VPA in late gestation

PPI: Low dose VPA exposure in late gestation did not disrupt preattentive stimulus filtering (PPI) or olfactory discrimination. Although PPI deficits have been reported in higher functioning adults with ASD [68] or Asperger’s Disorder [69], equivocal PPI findings have been reported in lower functioning children and adolescents [70] and in a cohort of individuals with both high- and low functioning individuals with ASD [71]. A higher dose of VPA in late gestation has been reported to alter PPI [67], however other late gestation exposures which may increase risk of ASD - specifically maternal immune activation - do not disrupt PPI either [28-30]. Thus, a PPI impairment is not always a consequence of exposure to ASD risks.

Olfactory function: To date, there have been few studies examining olfactory function in ASD patients. In animal models of ASD, studies which include direct assessment of olfactory function suggest that social differences may explain olfactory abnormalities [72]. For example, newborn mice treated with prenatal VPA have intact basic olfactory function, but changes in odor-based behaviors [73].

Thus, the absence of an effect of low dose VPA on PPI and olfactory processing does not negate prenatal exposure as of possible relevance to aspects of ASD.

Changes in the GABA system

GAD alteration in ASD: The behavioral alterations observed in our study were accompanied by widespread differences in GAD levels. Both isoforms of GAD, GAD67 and GAD65 are linked to ASD, but they are encoded by discrete, independently regulated genes (GAD1 and GAD2) and have distinct biochemical and cellular features [74]. GAD67 is present in readily detectable amounts in many GAD-containing cell bodies whereas GAD65 is particularly prominent in many axon terminals [75]. Here it appeared that prenatal VPA exposed had a ‘global’ impact upon both isoforms of GAD.

Differences in the expression of GAD isoforms have been reported in people with ASD: GAD65 and GAD67 has been reported to be lower in parietal and cerebellar

cortices [39]; GAD67 mRNA levels have been observed as lower in cerebellar Purkinje cells; GAD65 mRNA is lower in select subpopulations of neurons in the cerebellar dentate nuclei [76, 77]; and GAD67 mRNA expression is greater in cerebellar interneurons [78]. In the current study, animals exposed had lower GAD65 in all 6 brain regions examined; GAD67 was lower in MB, OB and PFC, but increased in CB, HP and STR. Thus, the pattern of a general reduction in levels of both GAD isoforms, but greater GAD67 in the cerebellum, fits with the picture reported in the human condition [78]. Hence, disruption to the GABA system may be common to VPA exposure and ASD, and consistent with hypotheses that an excitatory/inhibitory (E/I) imbalance may therefore contribute to altered information processing in both the clinical condition and preclinical model [38, 79].

Causes of GAD alteration in the VPA model: VPA may influence ASD risk in postnatal life via epigenetic mechanisms as it is a histone deacetylase inhibitor [80]. VPA-induced histone hyperacetylation could have multiple consequences. For example, in cultured rat cortical neurons, VPA-treatment up-regulated 726 genes and down-regulated 577 genes, including GAD65 and 67 [81]. The epigenetic effects of VPA during embryo development are likely to be even more complex however, as histone acetylation depends upon both brain region and developmental stage [82]. Thus the effects of late gestation exposure to VPA on GAD65 and GAD67 may be quite distinct from an earlier insult, but more work is needed to examine this.

Methodological considerations

VPA dose and gestation time of exposure: We chose a low dose of VPA compared to other studies of VPA model of ASD. In their first observations on the VPA mouse model of autism, Wagner et al [11] used 600mg/kg as a single s.c. injection on GD13, and 200mg/kg on six consecutive days. With this dose regime, only 9 out of 22 litters survived, compared to 8 out of 9 saline-treated litters. Consistent with this, in a recent report, an i.p. injection of 500mg/kg of VPA on GD9 induced obvious congenital malformations offspring at a rate of 30-40% [22]. In contrast, our results show that a single dose as low as 100mg/kg is sufficient to cause behavioural and biochemical abnormalities in offspring treated in utero with no overt effect on viability or malformation rate. A possible explanation for the impact of such a low dose VPA, may be VPA enrichment in especially in late gestation embryos. For example, after an s.c. injection of 550mg/kg VPA to pregnant mice, VPA concentration in embryo has been reported to be almost double of the concentration in the plasma of the dams [32]; and, for the same dose of VPA, the VPA concentration in embryos treated on GD7 is lower than in those treated on GD13 [32].

Sex: The behavioral phenotype and level of GAD in VPA mice was minimally influenced by sex. Sexual dimorphism has been observed in many psychiatric disorders. Early-onset disorders (e.g., autism) show a marked male bias while adolescent-onset disorders (e.g., depression, anxiety) show a marked female

preponderance [83]. Early gestation exposure to VPA in the rat (600 mg/kg VPA on GD12.5) produced more severe symptoms in males, including lower sensitivity to pain, increased repetitive/ stereotypic-like activity, higher anxiety and decreased social interaction; whereas females had only increased repetitive/stereotypic-like activity [12]. However, consistent with our findings ‘fetal anticonvulsant syndrome associated ASD’ is characterized by an even sex ratio [5].

Limitations: We acknowledge that our study has a number of limitations. These include a modest sample size. This was in order to minimize the animal numbers used. However, to make the optimal use of this sample, a strength of our study was that we attempted to examine the developmental course of offspring by testing the same animals at both young adult timepoints. The disadvantage of this approach is that we cannot completely rule out the possibility that, for behavioral tests conducted twice, exposure to the first tests may affect the results of tests conducted later in life. In addition, we could only collect GAD measures ex-vivo in adulthood, therefore we cannot say for sure that the alterations in GAD observed are present from a young age.

5. Conclusion

The present study evaluated the postnatal effect of low dose VPA exposure during late prenatal life in mice. This regime induced some subtle behavioral abnormalities

and caused widespread changes to GAD levels compared to saline treated controls.

Our results suggest that late gestation constitutes a period of vulnerability of the GABAergic system to environmental exposure in mice. Further work is needed to investigate if this observation translates to the human.

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